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The Homolog of the Gene *bstA* of the BTP1 Phage from *Salmonella enterica* Serovar Typhimurium ST313 Is an Antivirulence Gene in *Salmonella enterica* Serovar Dublin

Ana Herrero-Fresno,[a] Irene Cartas Espinel,[b] Malene Roed Spiegelhauer,[a] Priscila Regina Guerra,[a] Karsten Wiber Andersen,[a] John Elmerdahl Olsen[a]

[a]Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark

[b]Department of Pathological Anatomy, Faculty of Medicine, La Frontera University, Temuco, Chile

ABSTRACT In a previous study, a novel virulence gene, *bstA*, identified in a *Salmonella enterica* serovar Typhimurium sequence type 313 (ST313) strain was found to be conserved in all published *Salmonella enterica* serovar Dublin genomes. In order to analyze the role of this gene in the host-pathogen interaction in *S.* Dublin, a mutant where this gene was deleted (*S.* Dublin Δ*bstA*) and a mutant which was further genetically complemented with *bstA* (*S.* Dublin 3246-C) were constructed and tested in models of *in vitro* and *in vivo* infection as well as during growth competition assays in M9 medium, Luria-Bertani broth, and cattle blood. In contrast to the results obtained for a strain of *S.* Typhimurium ST313, the lack of *bstA* was found to be associated with increased virulence in *S.* Dublin. Thus, *S.* Dublin Δ*bstA* showed higher levels of uptake than the wild-type strain during infection of mouse and cattle macrophages and higher net replication within human THP-1 cells. Furthermore, during mouse infections, *S.* Dublin Δ*bstA* was more virulent than the wild type following a single intraperitoneal infection and showed an increased competitive index during competitive infection assays. Deletion of *bstA* did not affect either the amount of cytokines released by THP-1 macrophages or the cytotoxicity toward these cells. The histology of the livers and spleens of mice infected with the wild-type strain and the *S.* Dublin Δ*bstA* mutant revealed similar levels of inflammation between the two groups. The gene was not important for adherence to or invasion of human epithelial cells and did not influence bacterial growth in rich medium, minimal medium, or cattle blood. In conclusion, a lack of *bstA* affects the pathogenicity of *S.* Dublin by decreasing its virulence. Therefore, it might be regarded as an antivirulence gene in this serovar.

KEYWORDS *Salmonella enterica* serovar Dublin, *bstA*, antivirulence, infection, cell lines, cattle blood, mouse

More than 2,500 serovars of *Salmonella enterica* have been described so far, and several are important pathogens of animals and humans. All *S. enterica* serovars are closely related, and comparisons of their housekeeping genes show 96% to 99.5% nucleotide sequence identity (1). Despite this close relationship, serovars differ significantly in infection biology, specifically, in their host ranges and spectra of disease.

S. enterica strains may be widely divided into ubiquitous, host-adapted, and host-specific serovars (2). Host-specific serovars, such as *S. enterica* serovar Typhi, are almost exclusively associated with typhoidal disease in a single species (3, 4). Serovars which are mainly isolated from one particular host species but which sometimes cause illness in other host species are classified as host adapted. This is the case for the cattle-associated serovar *S.* Dublin, since it occasionally infects sheep and humans and is

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Address correspondence to Ana Herrero-Fresno, ahefr@sund.ku.dk.

I.C.E. and M.R.S. contributed equally to this article.

highly pathogenic in mice (5). Both host-specific and host-adapted serovars tend to cause septicemia with high mortality rates (2, 6).

Ubiquitous serovars encompassing nontyphoidal serovars (NTS), such as *S*. Typhimurium and *S*. Enteritidis, are frequently associated with self-limiting gastroenteritis in a broad range of species (5, 7). However, in underdeveloped countries, such as those in Africa, NTS (mostly *S*. Typhimurium and *S*. Enteritidis) are frequently the cause of invasive infections and meningitis with mortality rates of between 20% and 45%, more commonly in children with underlying diseases (such as malaria, anemia, or malnutrition) and in immunosuppressed HIV-infected adults (8, 9). Among the NTS currently circulating in Africa, a novel multidrug-resistant lineage, *S*. Typhimurium sequence type 313 (ST313), has emerged and is infrequently reported outside that continent (8–11).

Analysis of the genetic makeup responsible for differences in pathogenicity within and among *Salmonella* serovars represents a major research area. Therefore, in an attempt to understand the increased pathogenicity of the lineage *S*. Typhimurium ST313, the genomes of strains of that lineage were compared to those of other *S*. Typhimurium strains which usually cause gastroenteritis. Comparative genomics showed genomic degradation due to pseudogene formation, chromosomal deletions, and acquisition of a novel repertory of prophage-like elements (9–11). Pseudogene accumulation has been suggested to be associated with adaptation to the human host (12), and ST313 strains may thus be in the process of adapting to a host-restricted lifestyle, which may explain their high levels of association with systemic disease. In accordance with this theory, several clone-specific insertions, deletions, and frameshift mutations like those previously observed in host-specific serovars have been described in ST313 strains (11, 13–19).

In a previous study, we demonstrated the presence of a gene, *bstA* (previously termed *st313-td*), in strains of ST313 (9). The 924-bp gene is harbored in a novel gene island (ST313-GI) of approximately 17.7 kb (9, 10). Recently, this island has been shown to be part of a prophage, BTP1 (20). Interestingly, *bstA* was also found to be present in all the publicly available genomes of the highly invasive serovar *S*. Dublin, as well as in all *S*. Dublin strains from a collection of 50 isolates originating from cattle and humans (10). In *S*. Dublin, however, only a region of approximately 6.8 kb out of the 17.7-kb ST313-GI was detected.

The gene *bstA* is predicted to encode a protein whose function has not yet been determined. The gene was classified as a virulence gene, since its deletion led to the significantly decreased survival of a strain of *S*. Typhimurium ST313 within macrophages and decreased virulence in a mouse model of infection (10). Building on this observation, the aim of the present study was to analyze the role of *bstA* in the virulence of *S*. Dublin through the construction of a deletion mutant. The ability of this mutant to cause infection compared to that of the wild type (WT) was determined during infection of cell lines and infection of mice. Also, the growth performance of both strains during growth competition assays in rich and minimal media as well as in cattle blood was analyzed.

RESULTS

Bioinformatics analyses. The predicted protein encoded by the gene *bstA* was found to have homologs in strains of other pathogenic species: *Klebsiella pneumoniae* and *Cronobacter sakazakii* (95% identity). Single nucleotide polymorphism (SNP) analysis of *bstA* and the 400-bp region upstream and downstream of the gene revealed no SNP differences between *S*. Dublin and *S*. Typhimurium ST313 strains. In *S*. Dublin, *bstA* was harbored in an approximately 6.8-kb region that showed 99% identity to the same region of the ST313-GI in a strain of *S*. Typhimurium ST313 (10). The genetic structure of this region is shown in Fig. 1. Among other differences between the two serovars, the genes *cI* and *cro*, which surround *bstA* and which encode a phage repressor and antirepressor protein in ST313-GI, respectively, were absent from the 6.8-kb *bstA*-containing region in *S*. Dublin (Fig. 1).

Growth competition in M9 medium, LB broth, and cattle blood. Results from experiments in a Bioscreen C instrument revealed that both *S*. Dublin WT strain 3246

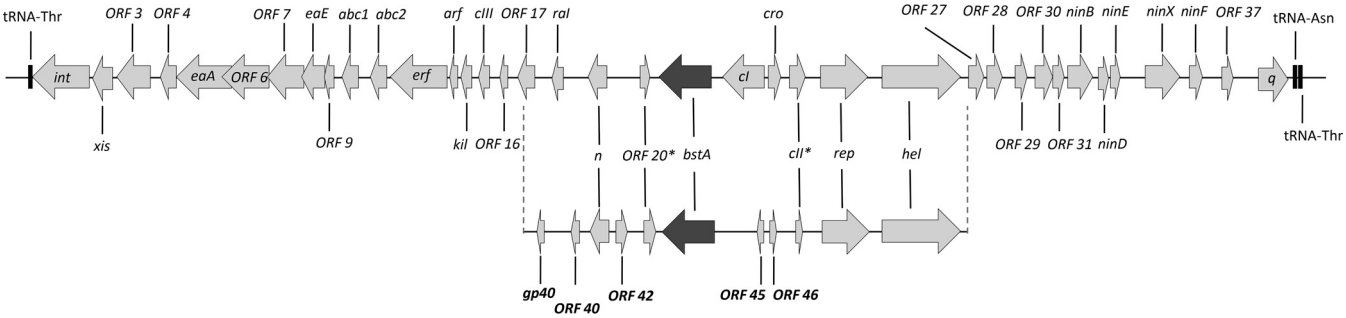

FIG 1 Genetic structure of ST313-GI of *S.* Typhimurium (top) and the 6.8-kb *bstA*-containing region (bottom) of *S.* Dublin. The sequence data were extracted from the sequenced genomes of strains *S.* Typhimurium D23580 and *S.* Dublin CT_02021853 (GenBank accession numbers FN424405 and CP001144, respectively). The location of the region of approximately 6.8 kb in the *S.* Dublin genome is positions 624455 to 631264. The *bstA* gene is highlighted in dark gray. Most of the remaining open reading frames show phage-associated functions: *int*, integration; *xis*, excision; *eaA* and *eaE*, Ea region; *abc1*, *abc2*, *arf*, *erf*, and *kil*, recombination; *cIII*, regulatory; *ral* and *n*, antitermination; *cI*, repressor; *cro*, antirepressor; *cII*, transcription; *rep*, replication; *hel*, helicase; *ninBDEXF*, *nin* region; *q*, antitermination. Open reading frames in bold are exclusively found in *S.* Dublin. *, only a part of these open reading frames is conserved in *S.* Dublin.

and the mutant *S.* Dublin Δ*bstA* showed similar growth performance characteristics in M9 medium and Luria-Bertani (LB) broth (not shown).

We also analyzed the growth ability of the WT and the mutant in M9 medium (Fig. 2), LB broth (not shown), and cattle blood during growth competition assays (Fig. 2). No differences between the two strains were observed in these assays, showing that the deletion of *bstA* did not affect the bacterial growth performance in any of the media. As expected from previous observations (21), the counts dropped for the first approximately 6 h in blood.

Uptake and survival in murine, cattle, and human macrophages. The ability of the WT strain *S.* Dublin 3246, the mutant *S.* Dublin Δ*bstA*, and strain *S.* Dublin 3246-C (which is *S.* Dublin Δ*bstA* complemented with *bstA*) to be taken up by and replicate

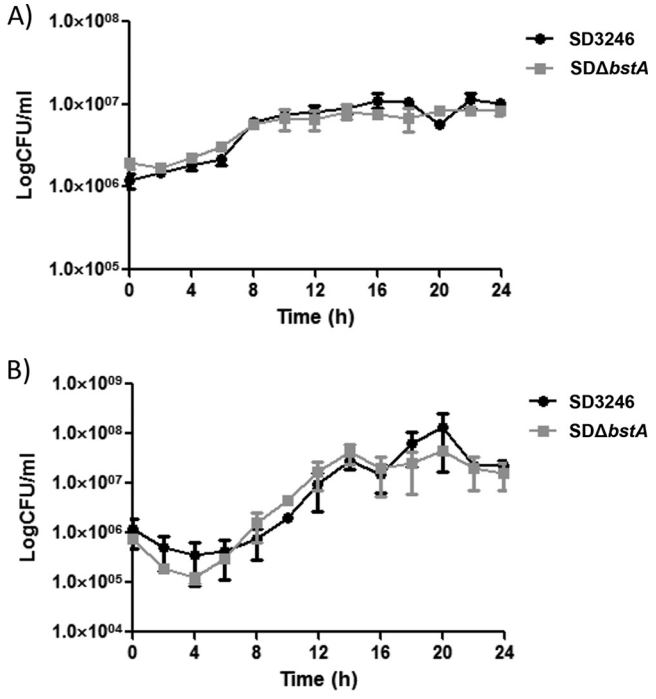

FIG 2 Growth of *S.* Dublin 3246 (SD3246) and the derived mutant strain *S.* Dublin Δ*bstA* (SDΔ*bstA*) under direct competition in different media: M9 medium (A) and cattle blood (B). The number of viable bacteria was determined by performing serial dilutions, which were plated on LB agar and LB agar supplemented with apramycin, every second hour from 0 to 24 h postinoculation. Data are the means ± SDs from three experiments. Statistical significance was determined by a paired *t* test.

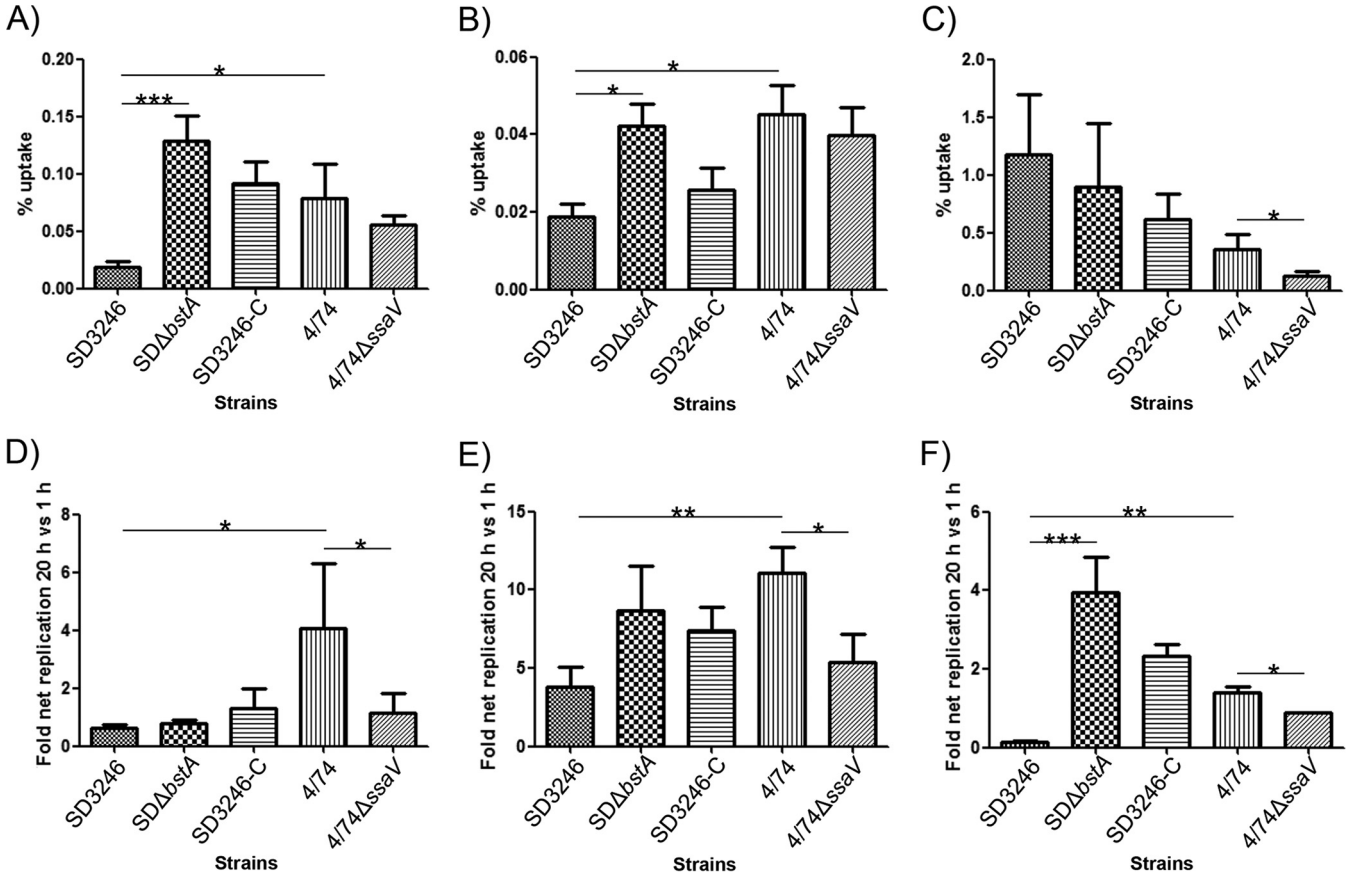

FIG 3 Interaction of *S*. Dublin 3246 and derived mutant strains with cultured macrophage cells. (A to C) Percentage of the challenge dose taken up by J774A.1 murine macrophages (A), BoMac bovine macrophages (B), and THP-1 human macrophages (C) at 1 h postinfection; (D to F) net replication within J774A.1 murine macrophages (D), BoMac bovine macrophages (E), and THP-1 human macrophages (F) at 20 h postinfection with regard to that at 1 h after uptake. The isolates analyzed were *S*. Dublin 3246 (SD3246), *S*. Typhimurium 4/74 (4/74), and the mutant strains *S*. Dublin Δ*bstA* (SDΔ*bstA*), *S*. Dublin 3246-C (SD3246-C; *S*. Dublin Δ*bstA* complemented with *bstA* via pAHF4), and 4/74 Δ*ssaV*. The assays were performed at least 4 times with similar results each time, and the results shown are averages from these assays. Error bars indicate standard deviations. Statistical significance (***, $P < 0.0001$; **, $P < 0.001$; *, $P < 0.05$) was determined by one-way ANOVA with pairwise comparison of the means.

inside cells of a mouse-derived macrophage-like cell line (J774A.1), cells of a bovine macrophage-like cell line (BoMac), and THP-1 macrophages was analyzed. At 1 h postinfection, a significantly higher percentage of uptake by mouse and cattle macrophages was detected for the mutant *S*. Dublin Δ*bstA* than for the WT strain *S*. Dublin 3246 ($P < 0.05$). This difference could partly be complemented by introducing the gene *bstA* back into the mutant strain, producing strain *S*. Dublin 3246-C (Fig. 3A and B). No significant difference in the net replication between *S*. Dublin Δ*bstA* and the WT strain was observed in the J774A.1 or BoMac cell infection assays (Fig. 3D and E), although in cattle cells the mutant appeared to survive better than the WT (Fig. 3E). When they infected human macrophages, similar uptake levels were obtained for the WT strain and the mutant *S*. Dublin Δ*bstA* (Fig. 3C), but a significantly higher net replication was observed for the mutant (Fig. 3F). These results are in contrast to those observed for a strain of *S*. Typhimurium ST313, where the mutant lacking *bstA* showed a significantly reduced ability to multiply inside macrophages (10).

The strain *S*. Typhimurium 4/74 Δ*ssaV* showed a significant decrease in net replication compared to that of the WT 4/74 strain ($P < 0.05$), as expected (Fig. 3D to F). We also observed a significantly higher net replication for the control strain of *S*. Typhimurium, WT strain 4/74, than for the WT *S*. Dublin 3246 isolate in all the infection assays ($P < 0.05$) (Fig. 3D to F).

No difference in cytotoxicity toward THP-1 macrophages between the WT strain and the mutant *S*. Dublin Δ*bstA* was observed. In general, the levels of lactate dehydroge-

TABLE 1 Competitive indexes for *S.* Dublin 3246 mutants in mice[d]

Salmonella strain (*n*,[a] group) compared with *S.* Dublin 3246	CI
S. Dublin Δ*bstA* (6, group 5)	1.55 ± 0.55[b]
S. Dublin 3246-C (6, group 6)	0.71 ± 0.13[c]
S. Dublin 3246/pACY177 (6, group 7)	0.61 ± 0.52

[a]*n*, number of mice in the experiment. All 6 mice from group 7 survived to the end of the experiment. One animal each from group 5 and group 6 was sacrificed 1 day before the end of the trial (4 days postinfection) (see Table SA1 in the supplemental material). Data for sacrificed mice were also included in the study.
[b]The CI was significantly different from 1.0 ($P < 0.05$).
[c]The CI was significantly different from that for the corresponding mutant ($P < 0.001$).
[d]Competition indexes were calculated on the basis of the input (number of CFU per milliliter of inoculum) and output (number of CFU per milliliter of spleen sample) numbers of WT versus mutant bacteria as previously described (10). The results are shown as mean values on the basis of the number of mice tested (indicated in parentheses in the first column). Statistical significance was determined by one-way ANOVA with pairwise comparison of the means.

nase (LDH) released into the supernatant of the cultured cells at 20 h postinfection were similar for all strains tested (see Fig. SA2 in the supplemental material).

Cytokine expression by infected macrophages. The release of the cytokines tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and IL-8 by activated TPH-1 macrophages at 4 h postinfection was estimated by quantitative reverse transcription-PCR (qRT-PCR). No significant differences in the amounts of any of the cytokines secreted by WT- and *S.* Dublin Δ*bstA*-infected macrophages were observed, although a slight increase in the amount of both IL-1β and IL-8 released was observed when the cells were infected with the mutant *S.* Dublin Δ*bstA* (Fig. SA3).

Adhesion and invasion of epithelial cells. Next, we tested the role of *bstA* in the adhesion to and invasion of a human epithelial cell line previously analyzed with a strain of *S.* Typhimurium ST313 (10). As demonstrated for the latter, deletion of the *bstA* gene did not affect *S.* Dublin adhesion to or invasion of cells of human epithelial cell line Int-407 (Fig. SA4). As expected, the control *invH201*::Tn*phoA* strain showed significantly decreased rates of adhesion and invasion ($P < 0.01$) than WT strain 4/74 (Fig. SA4). Both WT strains, *S.* Dublin 3246 and *S.* Typhimurium 4/74, showed similar rates of adhesion and invasion (Fig. SA4).

Mouse infections. In order to investigate whether *bstA* plays a role in the virulence of *S.* Dublin, *S.* Dublin Δ*bstA* was tested in competition with the WT strain in a mouse model of systemic infection. Notably and contrary to the results observed for a strain of *S.* Typhimurium ST313 (10), the lack of *bstA* increased the virulence of *S.* Dublin during mixed infections of C57BL/6 mice. Thus, the mutant *S.* Dublin Δ*bstA* outcompeted the WT (Table 1). Introduction of the plasmid pAHF4 (encoding a cloned copy of *bstA*) (10) into the mutant in *trans* partly restored the virulent phenotype to the WT level and eliminated significant differences between the strains (Table 1). The strain *S.* Dublin 3246/pACY177 (containing plasmid pACY177, which was used for cloning) was shown to compete evenly with the WT (Table 1). These results show that *bstA* decreases the virulence of *S.* Dublin, at least during systemic infection of mice, and that the presence of the plasmid pACY177 did not affect the WT phenotype.

The results of the mixed infection were further assessed by the use of single infections to rule out the possibility that cross talk between strains affected the outcome of the infection. In these experiments, the mutant *S.* Dublin Δ*bstA* showed a significantly higher number of CFU in the liver and the spleen than the WT, while both strains showed similar numbers of CFU in the mesenteric lymph nodes (MLN) (Fig. 4A).

The ratios of the weights of the spleens and MLN from mice infected with *S.* Dublin Δ*bstA* to the weights of the animals were significantly higher than the same ratios obtained for spleens and MLN collected from the WT-infected mice (Fig. 4B). However, similar degrees of cellular infiltration (liver and spleen) and a similar presence of focal necrosis (liver) were observed between the two groups of infected animals (Fig. SA5). We did not observe any pathomorphological findings in organs collected from noninfected mice (Fig. SA5).

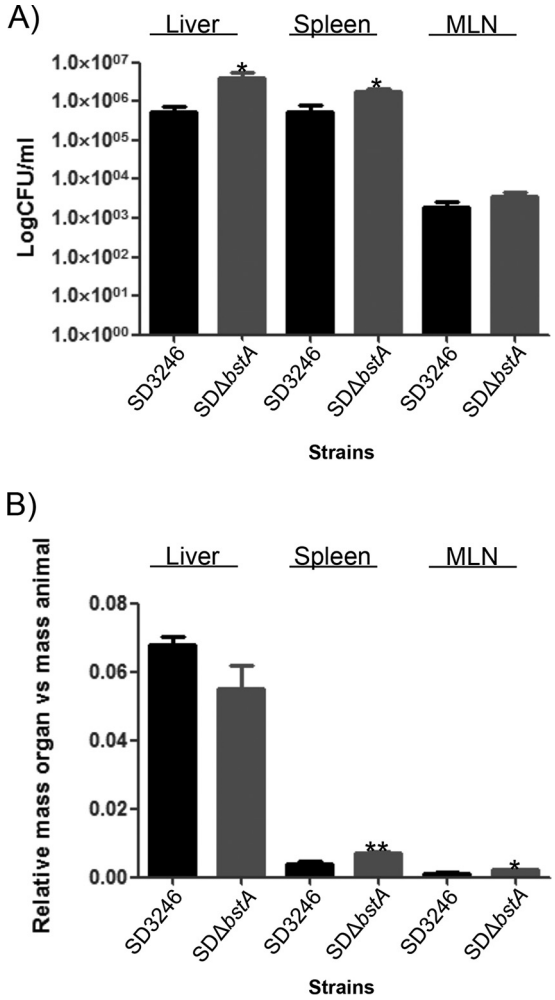

FIG 4 Single infection of mice with *S.* Dublin 3246 (SD3246) and the derived mutant strain *S.* Dublin Δ*bstA* (SDΔ*bstA*). (A) The log number of CFU per milliliter detected for each of the strains in the liver, spleen, and mesenteric lymph nodes (MLN); (B) enlargement of the liver, spleen, and MLN infected with each of the strains. Statistical significance (**, $P < 0.001$; *, $P < 0.05$) was determined by one-way ANOVA with pairwise comparison of the means (for the log number of CFU per milliliter) or paired *t* test (for enlargement of the organs).

DISCUSSION

S. Dublin is a host-adapted pathogen of cattle. In humans, it can cause septicemia with high rates of mortality. Infections are often caused by the consumption of contaminated raw milk (2, 6, 22, 23).

In a previous work, we demonstrated that the gene *bstA*, first detected in strains of the very invasive *S.* Typhimurium ST313 lineage, was also present in all publically available *S.* Dublin genomes as well in a collection of strains belonging to this serovar (10). Therefore, *bstA* has been suggested to be horizontally transferred from *S.* Dublin (the original host, in which it is apparently conserved) to *S.* Typhimurium ST313 strains (10). In support of this, in *S.* Typhimurium ST313 strains, the gene is harbored in a gene island, designated ST313-GI (10), that is part of a prophage (20). In *S.* Dublin, even though the gene is not harbored in the same gene island, it was found to be located in a region of approximately 6.8 kb with 99% identity with a part of ST313-GI and, in turn, is surrounded by phage-related genes.

The goal of the present work was to study the role of *bstA* in the virulence of *S.* Dublin. On the basis of our previous observations from *S.* Typhimurium ST313 strains, we expected that a mutant strain of *S.* Dublin lacking *bstA* would show reduced

virulence. Surprisingly, our data demonstrated that the inactivation of *bstA* enhanced virulence in *S.* Dublin, where it should be therefore considered an antivirulence gene.

Results derived from infection of an epithelial cell line suggested that the gene is not involved in adhesion to or invasion of the epithelium, which is similar to what has been shown for *S.* Typhimurium ST313 strains (10). On the other hand, it was shown to be important for uptake by macrophages and survival within these cells. Thus, in the absence of the gene, the mouse and cattle macrophage cell lines showed significantly increased levels of uptake of the bacteria. Once inside the macrophage, the net replication was not affected in the mouse cells; however, in the cattle and human macrophages, the *S.* Dublin Δ*bstA* mutant appeared to survive better than the WT and clearly showed a higher significant net replication, respectively. These results are contrary to the results obtained for the strain of *S.* Typhimurium ST313 previously tested, where *bstA* was found to enhance replication within macrophages (10).

Similarly, results from single and mixed infections in mice showed that the lack of the gene led to a significant increase in the level of virulence during systemic infection. This observation, too, was contrary to what was observed for *S.* Typhimurium ST313 strains (10). Thus, mice infected with the *S.* Dublin Δ*bstA* mutant showed a significantly higher number of bacteria in the liver and the spleen and a significant enlargement of the spleens and MLN compared to WT-infected mice. Nevertheless, no detectable differences in levels of inflammation (necrosis or cellular infiltration) were observed between organs collected from the two groups of mice.

Based on the results, we conclude that *bstA* is an antivirulence gene in *S.* Dublin. The apparent opposite functions of the gene in the two serovars of *Salmonella* is not an uncommon phenomenon, as other genes have been shown to have opposite functions in different bacteria (24–27). Also, there are previous examples of such a diverging phenotype between the two serovars compared in the current study. For instance, Olsen et al. (27) demonstrated that flagellum and chemotaxis genes differed in their role in the host-pathogen interaction between *S.* Dublin and *S.* Typhimurium. Interestingly, the absence of flagella led to a more virulent *S.* Typhimurium phenotype at systemic sites in the host, while this was not observed for *S.* Dublin.

In another study, the gene *grvA*, which, like *bstA*, is prophage associated (it is located in Gifsy-2) in *S.* Typhimurium, was characterized as an antivirulence factor (28). Mutants lacking *grvA* were more virulent than the WT during intraperitoneal infection of mice. Interestingly, the antivirulence phenotype resulting from the lack of *grvA* required the presence of WT *sodC1* (a well-known virulence gene in Gifsy-2), with *sodC1* being epistatic to *grvA*. A similar role could be attributed to *bstA* in this study, but further studies are needed to understand the interplay between genes. As suggested for GrvA, BstA might be a regulator of virulence, affecting the expression of a particular or several virulence genes (as shown for *grvA*) (28). In order to test this hypothesis and to clarify the specific function of BstA, studies that use various analytical approaches, including mutagenesis, transcriptomic, and proteomic studies, should be carried out. In a previous study, *bstA* was found to be present in other *Salmonella* serovars, such as *S.* Bredeney, *S.* Saintpaul, and *S.* Kentucky (9). Therefore, appropriate infection assays should be also performed to investigate the association of the gene with the virulence of these serovars. Of relevance to the current study, we have recently demonstrated that the lack of *bstA* in *S.* Typhimurium D23580, the reference strain for the ST313 lineage, led to results similar to those observed in this study for *S.* Dublin (unpublished results). Thus, a *bstA*-deficient mutant showed better survival within macrophages and was more virulent during the systemic infection of mice. Since these data contradict our previous results (10), further studies to understand the divergent role in different strains of ST313 are also warranted.

Currently, it is not fully understood why bacteria need antivirulence genes. It has been speculated that pathogens might naturally evolve toward a less virulent phenotype to ensure that the pathogen does not kill the host and thus eliminates the basis for its own propagation (29, 30). In this context, the lower levels of virulence observed

TABLE 2 Strains, derived isogenic isolates, and plasmids used in the present work

Strain or plasmid	Relevant features[a]	Reference or source
Salmonella strains		
3246	*S.* Dublin wild-type, *bstA*-positive strain	22
4/74	*S.* Typhimurium wild-type, virulent reference strain	23
KP1274	*Escherichia coli* restriction-deficient strain	38
Δ*bstA*	*S.* Dublin 3246 lacking *bstA*, Apr^r	This work
3246-C	*S.* Dublin Δ*bstA* complemented with *bstA* by pACY177 + *bstA*::pAHF4, Apr^r Kn^r	This work
3246/pACY177	*S.* Dublin 3246 containing pACY177	This work
invH201::Tn*phoA*	4/74 lacking *invH*, Kn^r	35
4/74 Δ*ssaV*	4/74 lacking *ssaV*, SPI2-T3SS defect, Kn^r	This work
Plasmids		
pACY177	Cloning vector, Ap^r Kn^r	39
pAHF4	pACY177 expressing *bstA*, Kn^r	10
pKD46	Plasmid with bacteriophage λ red recombinase expressed from an arabinose-inducible promoter, Ap^r	32
pUO9090	Apr^r	Unpublished
pKD4	Kn^r	32

[a]Apr^r, Kn^r, and Ap^r, apramycin, kanamycin, and ampicillin resistant, respectively.

for NTS of *Salmonella* than for typhoidal serovars represent an example of evolution toward decreased virulence (29).

Since *S.* Dublin is adapted to cattle, in which it causes systemic disease, *S.* Dublin 3246 and the mutant strain *S.* Dublin Δ*bstA* were grown in direct competition in cattle blood. No significant difference was observed between them, suggesting that *bstA* is not important for growth in the extracellular environment during systemic disease of cattle. Infections of calves with the WT and isogenic strains should be performed in order to analyze the role of *bstA* in virulence in the calf model of infection.

In our study, the phenotype conferred by *bstA* is clearly reproducible and statistically significant. In order to gain a full understanding of the disease process, it is of paramount importance to understand and clarify the roles of these kinds of factors.

MATERIALS AND METHODS

Ethics statement. Experiments with mice were conducted according to the principles expressed in the Declaration of Helsinki. Mouse infection studies were performed with the permission of the Danish Animal Experiments Inspectorate (license number 2009/561-1675).

Bioinformatics analyses. Information available at NCBI (www.ncbi.nlm.nih.gov) and in the UniProt database (www.uniprot.org) was used to investigate the presence of homologs of *bstA* in non-*Salmonella* bacteria and to increase our knowledge of the putative role of the protein encoded by *bstA*. The sequence of the gene island from *S.* Typhimurium ST313 (ST313-GI) strain D23580 (GenBank accession number FN424405) was compared with the sequence of the genome of *S.* Dublin CT_02021853 (GenBank accession number CP001144) by BLAST analysis (31) to find areas in common between the two genomes, and the genetic structure of the approximately 6.8-kb *bstA*-containing region in *S.* Dublin was established. The CLC sequence viewer (version 7.7; Qiagen) was used to search for single nucleotide polymorphisms (SNPs) in the gene *bstA* and the region immediately surrounding *bstA* (400 bp upstream and downstream of the gene) between the two serovars.

Bacterial strains and growth conditions. *S.* Dublin 3246 was used as the wild-type (WT) *bstA*-positive strain (Table 2) in all the experiments. This strain was isolated from a case with bovine septicemia (22). The *S.* Typhimurium ST19 WT strain 4/74 (which does not harbor *bstA*) and its derived mutants, the *invH201*::Tn*phoA* and 4/74 Δ*ssaV* mutants (Table 2), were used as controls in the *in vitro* infection assays. The virulence properties of 4/74 are well-known (23). The isolates were propagated in Luria-Bertani (LB) broth (Oxoid) overnight at 37°C with shaking at 200 rpm. The LB medium was supplemented where appropriate with kanamycin or apramycin at 25 μg/ml (Sigma). Samples from the overnight cultures obtained were used to produce inocula in all the experiments.

Mutagenesis techniques. The gene *bstA* was disrupted in the strain *S.* Dublin 3246 by using the bacteriophage lambda Red-mediated recombination technique (32), resulting in the isogenic strain *S.* Dublin Δ*bstA*. The selection of mutants was performed on LB agar plates supplemented with apramycin (75 μg/ml) as previously described (10). The apramycin resistance-encoding gene [*aac(3)-IV*] was amplified from plasmid pUO9090 (M. C. Martín, unpublished results). The absence of the gene *bstA* in the *S.* Dublin Δ*bstA* strain was confirmed by PCR using the primers and conditions described previously (10). For genetic complementation, the recombinant pACY177-derived plasmid containing the gene *bstA*, pAHF4, was purified and electroporated into the competent strain of *S.* Dublin Δ*bstA* in order to obtain

the complemented strain *S.* Dublin 3246-C, also as previously described (10). A mutant strain of *S.* Dublin 3246 containing the empty vector pACY177 (*S.* Dublin 3246/pACY177) was also constructed and used as a control in the mouse challenge experiments. The mutant strain 4/74 Δ*ssaV*, lacking the gene *ssaV*, was also obtained by following the procedure mentioned above. The kanamycin resistance-encoding gene was amplified from the plasmid pKD4 (32). The WT strains and plasmids used for the mutagenesis methods as well as the resulting isogenic strains are described in Table 2.

Analysis of the growth of WT and mutant strains. The WT and the mutant strains were compared for their ability to grow in LB and M9 media as previously described (10). Briefly, the overnight cultures were subcultured into fresh medium at a 40-fold dilution, and further growth was assessed every 15 min for 18 h using a Bioscreen C instrument. A growth curve for each of the strains was obtained, and comparison of the growth was performed visually on the basis of the optical density (OD) values detected for each strain at the different time points tested. *S.* Dublin 3246 and the isogenic strain *S.* Dublin Δ*bstA* were also cocultured in M9 medium, LB medium, and fresh cattle blood as previously described (21). The isolate *S.* Dublin Δ*bstA* was resistant to apramycin, allowing its differentiation from the WT. The overnight cultures were inoculated at a final concentration of approximately 10^6 CFU/ml (1:1 ratio) and incubated at 37°C with shaking for 24 h. Samples were collected right after inoculation and every second hour for a period of 24 h, and 10-fold dilutions were plated on LB agar plates with and without apramycin (75 μg/ml). The plates were incubated overnight at 37°C, and counting of the number of CFU was performed. The experiment was performed in triplicate.

Infection of mouse, cattle, and human macrophages. The role of the gene *bstA* in intracellular survival and replication within mouse, cattle, and human macrophages was investigated using cells of a mouse-derived macrophage-like cell line (J774A.1), a bovine macrophage-like cell line (BoMac), and a human monocytic cell line (THP-1) as previously described (10, 33) with modifications. Briefly, the cells were cultured in RPMI–GlutaMAX-I, Earle's salts, 25 mM HEPES (Gibco) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, and 25 μg/ml gentamicin. WT strain 4/74 and the isogenic 4/74 Δ*ssaV* strain were used as controls. The 4/74 Δ*ssaV* mutant lacks the gene *ssaV*, which encodes a structural component of the SPI2-encoded type III secretion system (T3SS), leading to a decreased rate of intracellular replication (34). Cells were incubated in a humidified 37°C incubator with a 5% CO_2 atmosphere. THP-1 cells (2×10^6) were seeded in 12-well plates and activated with 50 μg/ml phorbol 12-myristate 13-acetate (PMA) for 48 h before the infection experiments. Bacteria grown to exponential phase were added at a multiplicity of infection (MOI) of between 10:1 and 100:1 (ratio of bacteria/cells, which was precisely determined for each experiment). The cell monolayers were centrifuged at 300 rpm for 5 min at room temperature immediately after addition of the bacteria, followed by incubation for 30 min at 37°C in a 5% CO_2 atmosphere. The numbers of bacteria in the inoculum (the CFU counts before infection) were verified by plating onto LB agar plates. After the infection time, the medium was removed and the cell monolayers were washed twice with Dulbecco's phosphate-buffered saline (DPBS; Gibco). At this point, fresh medium containing 250 μg/ml amikacin was added to kill the extracellular bacteria. The plates were then incubated for a further 30 min at 37°C in a 5% CO_2 atmosphere before new medium supplemented with 100 μg/ml amikacin was added for the remaining part of the experiment. To enumerate the bacteria, cells were washed twice with DPBS and subsequently lysed in 1 ml 0.1% (vol/vol) Triton X-100. The viable intracellular bacteria were enumerated by determination of the colony counts from lysate dilutions plated on LB agar plates. The percentage of bacteria taken up by macrophages (percent uptake) was calculated by dividing the number of bacteria inside the cells (at 1 h postinfection) by the number of bacteria in the inoculum and multiplying by 100. Values for intracellular bacteria determined at 4 h postinfection (only for THP-1 macrophages) and 20 h postinfection were expressed relative to the number of CFU detected for the specific strain at 1 h postinfection. Each strain was tested in duplicate wells in at least four biological repeats.

Cytotoxicity assays. The cytotoxicity of the strains toward activated THP-1 macrophages was estimated by measuring the release of cytosolic lactate dehydrogenase (LDH) into the cell culture supernatants using a colorimetric CytoTox 96 kit (Promega) at 20 h postinfection as described previously (10). The relative amount of LDH released was calculated as $100 \times$ [(experimental release − spontaneous release)/(total release − spontaneous release)], where experimental release is the amount of LDH activity in the supernatant of infected cells, spontaneous release is the amount of LDH activity in the supernatant of uninfected THP-1 cells, and total release is the activity in cell lysates.

Infection of epithelial cell lines. The association between the gene *bstA* and the infection of the human epithelium was analyzed using Int-407 cells (HeLa cell-derived epithelial cells) as previously described (10). WT strain 4/74 and the isogenic *invH201*::Tn*phoA* strain were used as controls for the infection assays. The latter has a mutation in the *invH* gene, reducing its rate of invasion approximately 1 $\log_{10}$ unit compared to that of the WT strain (35). The cells were grown statically in a humidified 37°C incubator with a 5% CO_2 atmosphere. At 24 h prior to infection, the Int-407 cells were seeded in 24-well plates at a concentration of 2.5×10^5 cells per well and left under the conditions mentioned above. The bacteria were grown for 16 h at 200 rpm and 37°C in LB medium, subcultured into fresh LB medium, and incubated for an additional 3 h. At this point, when the bacteria reached an OD at 600 nm (OD_{600}) of approximately 0.5, the cultures were centrifuged at 6,000 rpm for 5 min. The bacteria were resuspended to an OD_{600} of 1.0 in DPBS and added to the monolayers at a multiplicity of infection of approximately 100:1. The counts of bacteria in the inocula were verified by plating on LB agar plates. After 1 h of infection, the medium was removed and the cell monolayers were washed twice with DPBS. At this point, adhesion was estimated, and for assessment of invasion, fresh medium containing 250 μg/ml amikacin was added to the remaining wells to kill the extracellular bacteria and the plates were incubated for a further 2 h at 37°C in a 5% CO_2 atmosphere. To enumerate the bacteria that adhered to the cells (1 h

TABLE 3 Primers used for quantitative PCR

Gene name	Orientation[a]	Primer sequence (5′-3′)	Reference
TNF-α	FW	CCCCAGGGACCTCTCTCTAATC	40
	RV	GGTTTGCTACAACATGGGCTACA	
IL-1β	FW	CCTGTCCTGCGTGTTGAAAGA	40
	RV	GGGAACTGGGCAGACTCAAA	
IL-8	FW	GCAGCTCTGTGTGAAGGTGCAGT	41
	RV	GTGTTGGCGCAGTGTGGTCC	
PGK1	FW	AAGAACAACCAGATAACAAACAAC	42
	RV	GTGGCTCATAAGGACTACCG	
PPIB	FW	GTCCGTCTTCTTCCTGCTG	42
	RV	CATCTTCATCTCCAATTCGTAGG	

[a]FW, forward; RV, reverse.

postinfection) and the bacteria that invaded the cells (3 h postinfection), the cells were washed twice with DPBS and lysed in 1 ml 0.1% (vol/vol) Triton X-100. The viable intracellular bacteria were enumerated by determination of the colony counts from lysate dilutions plated on LB agar. The experiment was performed in quadruplicate.

Cytokine expression. Total mRNA was isolated from infected THP-1 cells at 4 h postinfection using an RNeasy minikit (Qiagen) according to the manufacturer's instructions. RNA was eluted in 35 μl RNase-free water, and samples were stored at −80°C. RNA was treated with DNase (RQ1 RNase-free DNase; Promega) to remove any traces of DNA (36). cDNA was obtained by reverse transcription (GoScript reverse transcriptase; Promega) using the following conditions in a PCR machine: 25°C for 5 min, 42°C for 59 min, and 70°C for 15 min. qRT-PCR was carried out using a LightCycler 96 real-time PCR system (Roche) utilizing the FastStart essential DNA green master mix (Roche) and transcript-specific primers (Table 3). mRNA expression profiles were normalized to the levels of the phosphoglycerate kinase 1 (PGK1) and peptidylprolyl isomerase B (PPIB) housekeeping genes in each sample (Table 3), and the fold change in expression was calculated by the $2^{-\Delta\Delta CT}$ threshold cycle (C_T) method (37).

Single and mixed infections in mice. Infections of 5- to 6-week-old female C57BL/6 mice were performed as previously described (10). Briefly, groups of six mice each were inoculated intraperitoneally with either 0.1 ml of DPBS (control), 0.1 ml of a single specific strain suspended in DPBS (single infections), or 0.1 ml of a 1:1 mixture of the WT and one of the mutant strains (mixed infections). Bacterial overnight cultures were harvested by centrifugation and resuspended in DPBS. The OD was measured and adjusted to an OD_{600} of 0.1, and 10-fold dilutions were prepared. For the single infections, the challenge dose was 5×10^3 bacteria. For the mixed infections, the WT strain and a mutant strain were mixed before the infection to provide a challenge dose which also contained 5×10^3 bacteria of each strain. The number of CFU and the ratio between the WT and mutant strains were estimated by plating 10-fold dilutions of each inoculum. Mice were killed at 4 days (single infections) and 5 days (competition studies) postinoculation by cervical dislocation. Severely affected animals were sacrificed earlier for animal welfare reasons but were otherwise treated like the rest of the group. The livers, spleens, and mesenteric lymph nodes (MLN) from the mice with single infections were removed aseptically, and the bacteria were recovered. Tenfold dilution series were prepared and plated on LB agar plates, and the numbers of CFU were determined. In the mixed infection assays, suspensions of spleens in DPBS were plated on LB agar, and 100 colonies were randomly picked and tested for resistance to the relevant antimicrobial compounds (apramycin at 75 μg/ml or kanamycin at 50 μg/ml) to determine the proportion of mutant strains. The competitive index (CI) was calculated on the basis of the ratio of mutant bacteria/WT bacteria from the spleen in relation to the ratio of mutant bacteria/WT bacteria in the inoculum. A CI of 1 indicates that the virulence of the strains tested is equal. A CI of <1 shows that the mutant is less virulent than the WT.

For the single infections, the weights of the animals and organs were determined prior to determination of the number of CFU using a calibrated digital balance (Sartorius).

All relevant information about the infected mouse groups is shown in Table SA1 in the supplemental material.

Histology. For histological examinations of tissue sections, the spleens and livers were collected from 15 mice which were singly inoculated with the WT strain ($n = 6$), the *S.* Dublin Δ*bstA* mutant ($n = 6$), or DPBS ($n = 3$, control). Lumps of the organs were fixed in 10% (vol/vol) neutral buffered formalin phosphate (Fisher Scientific) for 72 h and paraffin embedded. Sections (4 to 6 μm) were deparaffinized in xylol and rehydrated in ethanol (100%, 96%, 80%, and 70%), followed by rehydration for 2 min in distilled water for hematoxylin and eosin (H&E) staining. The slides were evaluated with an Olympus BX45 light microscope with an attached DP25 digital camera (B & B Microscopes Limited). Histological grading was performed by a certified veterinary pathologist at the University of Copenhagen.

Statistical analysis. For multiple comparisons, one-way analysis of variance (ANOVA) with a pairwise comparison of means calculated with Dunnett's comparison posttest was performed with the GraphPad Prism (version 5.1) program (GraphPad Software Inc.). The same statistical approach was used to

determine the significance of the competitive indexes (CI) obtained for the mouse infections. One-sample *t* test analysis was performed to evaluate statistical significance during the growth competition studies and for analysis of enlargement of the organs. A *P* value of less than 0.05 was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00784-17.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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